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PRINCIPAL INVESTIGATOR:Julia Etchin, M.S.

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Yale University
New Haven, CT06520

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14. ABSTRACT
In eukaryotes, homologous recombination and the homology-directed repair of DNA double-strand breaks are mediated by the RAD51 recombinase. In catalyzing recombination reactions, RAD51 must first form a right-handed helical filament, termed the presynaptic filament, on single-stranded DNA. Emerging evidence indicates that BRCA2 acts a recombination mediator by promoting the assembly of the RAD51 presynaptic filament. BRCA2 binds DNA and associates with RAD51. Our laboratory has established biochemical systems to examine the recombination mediator function of BRCA2. The main focus of my fellowship project is to define the role of DNA binding in this BRCA2 function. The BRCA2 DNA-binding domain (DBD) represents a highly conserved region within BRCA2-like molecules and harbors a significant portion of tumor-derived missense mutations, underscoring the importance of addressing the functional significance of this BRCA2 domain.

15. SUBJECT TERMS
DNA Repair; Tumor Suppressor

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Introduction

Germ line mutations in the breast cancer susceptibility gene BRCA2 predispose carriers to early-onset breast cancer. BRCA2-deficient cells exhibit chromosomal instability and increased sensitivity to genotoxic agents. The involvement of BRCA2 in DNA double-strand break repair through the homologous recombination pathway is likely to account for these phenotypic changes. However, the mechanistic role of BRCA2 in homologous recombination remains to be defined. The main goal of this fellowship project is to define the role of DNA binding in recombination mediator function of BRCA2. Furthermore, in the recent years the scope of our research extended to include biochemical characterization of BRCA2's nuclear partner PALB2/FANCN. Mutations in PALB2 are associated with genetic predisposition to breast cancer. PALB2 acts as a linker of BRCA1 and BRCA2 in DNA-damage response pathway, and enables BRCA2-mediated homologous recombinational repair. The results of our study demonstrate that PALB2 directly binds DNA and exhibits preference for structured DNA substrates over single stranded and double stranded DNA.

Body

To elucidate the relevance of DNA-binding by BRCA2 in the DNA homology-directed repair of chromosomal breaks and to delineate the effect of cancer mutations on this BRCA2 function, I conducted the following molecular studies as outlined in the Statement of Work of my fellowship proposal.

To delineate the mode and the specificity of DNA binding by BRCA2, I purified human BRCA2 DBD and defined its DNA binding properties. I examined the affinity of BRCA2 DBD for different length DNA substrates. I conducted DNA binding with purified BRCA2 DBD. In these experiments, ^{32}P-labeled ss DNA substrates free of secondary structure (e.g. poly dT) were individually incubated with several different amounts of BRCA2 DBD, followed by analysis of the reaction mixtures in a non-denaturing polyacrylamide gel. After drying, the gel was subject to phosphorimaging analysis to detect DNA binding. The results indicate that the minimal DNA binding region of BRCA2 DBD is 24 nucelotides (shown in previous report).

BRCA2 contains three canonical OB (oligonucleotide and oligosaccharide binding) folds that confer a DNA binding ability. Due to the exceedingly large size of human BRCA2 (3,418 amino acid residues), it has not yet been possible to obtain sufficient amounts of full-length protein for mechanistic studies. Thus, our research group employed a modular approach that entails combining selected BRC repeats that are known to bind RAD51 with avidity (Chen et al, 1998; Wong et al, 1997), with the DBD of BRCA2 (SanFilippo et. al., 2006).

To determine the importance of OB folds in DNA binding affinity by BRCA2, I designed constructs consisting of a RAD51 binding module (BRC4) fused to either individual OB folds or OB folds in tandem. I expressed these polypeptides fused to a six histidine tag in bacteria. I carried out a five-step procedure - encompassing ammonium sulfate precipitation of bacterial extract, an affinity step in nickel-NTA agarose, and also chromatographic fractionation in macro-hydroxyapatite (MHAP) column to purify

BRCA2 DBD variants. Although the purification scheme devised is optimal for these mutant polypeptides, the bacterial extract does not present the most suitable system for purification of BRCA2 DBD variants. Previously, a postdoctoral fellow in our laboratory successfully carried out expression and purification of BRCA2 BRC4 DBD in insect cell system. To determine the importance of OB folds in DNA binding affinity by BRCA2, I transferred BRC4 OB fold variants into vectors suitable for expression in insect cells. I expressed these polypeptides fused to GST and six-histidine tags. To this date, I successfully purified BRCA2 BRC4 DBD, BRC4 OB12, and BRC4 OB23 fold constructs to near homogeneity (Figure 1 in the Appendices). To determine the role of OB folds in DNA binding properties of BRCA2 DBD, I carried out electrophoretic shift mobility assay (EMSA) with the resulting polypeptides. Consistent with previously published data by SanFilippo et al., BRC4 DBD demonstrates a definite preference for ssDNA over dsDNA (SanFilippo et al., 2006, Figure 2 in the Appendices). BRC4 OB12 and BRC4 OB23 preserve a strong preference for ssDNA, but bind DNA species with slightly lower affinity (Figure 2 in the Appendices). I am currently working on purification of BRC4 OB1, BRC4 OB2, and BRC4 OB3 to determine the DNA binding capabilities of single OB folds. I am also testing BRC4 DBD, BRC4 OB12, and BRC4 OB23 polypeptides in *in vitro* homologous pairing reaction to determine the relevance of OB folds on recombination mediator function of BRCA2 (Figure 3 in the Appendices). In addition, I fused C-terminal region of BRCA2 (CTRB), which has been reported to be involved in stabilizing presynaptic RAD51 filament, to BRC4 OB fold constructs in order to test the importance of OB folds in the highly physiologically relevant settings. To this date, I successfully purified BRC4 OB1 CTRB, BRC4 OB12 CTRB, and BRC4 OB23 CTRB variants (Figure 4 in the Appendices). I will assay these polypeptides for DNA binding and recombination mediator activities.

A striking feature of the BRCA2 DBD is the Tower anchored on OB2. The Apex of the Tower is a three helix bundle that resembles the helix-turn-helix double-stranded DNA binding motif. We have shown that BRCA2 DBD is capable of binding double-stranded DNA. To investigate the contributions of the Tower domain to the DNA-binding activity of BRCA2, I expressed and purified constructs deleted for the Tower (delT) or the Apex of the Tower (delA) in the context of the BRCA2 BRC12 DBD CTRB. I carried out DNA binding experiments with the resulting polypeptides and observed a reduced binding affinity for double-stranded DNA by the polypeptide deleted for the Tower domain when compared to its wild type counterpart (Figure 5 in the Appendices). The preliminary results demonstrate that the deletion of the Apex of the Tower also attenuates dsDNA binding activity of BRCA2 BRC12 DBD CTRB.

The BRCA2 DBD represents a highly conserved region within BRCA2 orthologues and harbors a significant portion of cancer-derived missense mutations, emphasizing the importance of this region in the tumor suppressor function of BRCA2 (BIC database: Szabo et al, 2000). By delineating the DNA binding and recombination mediator activities of BRCA2, I hope to provide important information that will enhance our understanding of the effect of tumor-derived DBD mutations on BRCA2-dependent recombination.

Recently, Ashworth and Taniguchi groups published their research findings directed at understanding resistance to chemotherapy that is observed in BRCA2-inactivated cells (Edwards et al., 2008, Sakai et al., 2008). The anticancer drugs used in these studies, which are platinum analogs and poly(ADP-ribose) polymerase inhibitors, exploit the incapability of BRCA2-deficient cells to rely on HR for the repair of DSBs. Apparently, the molecular basis for the observed drug resistance is the production of shortened BRCA2 fragments that are HR-competent. The genomic sequencing of these BRCA2 isoforms revealed that the newly-formed BRCA2 variants contain internal deletions that lead to restoration of the BRCA2 ORF. The deletions observed in the mutant BRCA2 variants encompass the DBD of BRCA2, which is surprising given the importance of this evolutionarily-conserved domain in BRCA2's mediator function. Interestingly, the Livingston group identified a nuclear partner of BRCA2, PALB2, named for "partner and localizer of BRCA2 (Xia et al., 2006)." Our laboratory became interested in testing the hypothesis that PALB2 may possess its own DNA binding activity, and if so, to define the interplay between the DNA binding activities of BRCA2 and PALB2.

Together with the post-doctoral fellow in our laboratory, I expressed and purified PALB2 constructs and identified the DNA binding domain (DBD) within N-terminal region of PALB2 (Figure 6 in the Appendices). My project focused on detailed characterization of the DNA binding domain of PALB2 and on delineation of the minimal region required for PALB2's direct interaction with DNA. I tested PALB2 for binding to DNA structures that resemble recombination intermediates since the structure preference by PALB2 may hint at its function in homologous recombination. The results of electrophoretic mobility shift assays revealed that PALB2 DBD exhibits preference for binding to D-loop, Bubble, and Holliday Junction substrates over double-stranded and single-stranded DNA (Figure 7 in the Appendices). Furthermore, deletion mapping led to the identification of two DNA-binding sites within the N-terminal region of the protein, with opposing DNA-binding selectivity. The first 100 residues stretch of the protein prefers binding to single stranded DNA over double stranded DNA, whereas the region encompassed by residues 100 to 184 prefers interaction with duplex DNA over single stranded DNA (Figure 8). These results suggest the direct involvement of PALB2 in DNA processing during homologous recombination.

We are currently in the process of isolating PALB2 mutant that will disrupt its interaction with DNA. Once introduced in the full length protein, this mutant will be of great importance for exploring the functional significance of PALB2.

Key Research Accomplishments

Task 1: To delineate the mode and the specificity of DNA binding by BRCA2.

- Defined the minimal DNA length required for BRCA2 binding
- Fused BRC4 region to either individual OB folds or OB folds in tandem (to create BRC4 OB fold variants) for expression and purification in insect cell system
- Fused BRC4 OB fold variants to CTRB domain for expression and purification in insect cell system
- Devised purification scheme for the aforementioned BRCA2 BRC4 DBD and BRC4 DBD CTRB mutant polypeptides to near homogeneity
- Purified BRCA2 constructs deleted for the Apex of the Tower domain (delA) or the Tower domain (delT) in the context of the BRCA2 BRC12 DBD CTRB and carried out DNA binding experiments with these polypeptides
- Introduced the cancer-associated Tower mutations - E2856A, I2944F, K2950N, and A2951V - into BRC4 DBD constructs

Task 2: Define the relevance of the OB folds and the Tower domain on the recombination mediator function of BRCA2 and examine the effect of tumor-derived DBD mutations on this BRCA2 function.

- Purified human RAD51 and RPA proteins and confirmed their activity in recombination mediator assays previously established in the laboratory
- Carried out recombination mediator assays using the wild type BRC4-DBD polypeptide in order to optimize the reaction conditions for future assessment of BRC4 DBD and BRC4 DBD CTRB variants

Reportable Outcomes

Yale University provides an excellent training environment for breast cancer research, with a formal Breast Cancer Research Program (BCRP) within the Yale Cancer Center, a NCI-designated Comprehensive Cancer Center. As a component of my training, I presented my research findings in the seminar organized by BCRP and research-in-progress meetings in the Genetics and Molecular Biophysics and Biochemistry departments at Yale. I presented my poster at the Era of Hope Meeting in Baltimore, MD (June 25-28, 2008) and Keystone Symposium on Genome Instability and DNA repair in Taos, New Mexico (March 1-5, 2009).

I am a coauthor on manuscript “Enhancement of the RAD51 Recombinase by the Tumor Suppressor PALB2” which is currently being reviewed in Science.

Conclusion

The focus of my research is to delineate the importance of DNA binding by BRCA2 in its recombination mediator function. By characterizing the biochemical functions of BRCA2, we should gain a molecular understanding of why breast tumorigenesis is associated with BRCA2 mutations. The knowledge garnered from my studies could very well be exploited for the prevention, diagnosis, and treatment of breast cancer.

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Appendices

Figure 1. Purification of BRC4 DBD, BRC4 OB12, and BRC4 OB23 constructs. (A) Purification scheme for BRC4 DBD, BRC4 OB12, and BRC4 OB23**. (B)** SDS-PAGE of purified proteins.

Figure 2. DNA binding by BRC4 DBD, BRC4 OB12, and BRC4 OB23. Increasing concentrations (10 to 300nM in lanes 2 to 7 for BRC4 DBD, and 10 to 500nM in lanes 2 to 9 for BRC4 OB12 and BRC4 OB23) of indicated protein were incubated with the mixture of 10nM ^{32}P-labeled 80-mer ssDNA and dsDNA and resolved by non-denaturing polyacrilamide gel.

Figure 3. Recombination mediator activity of BRC4 DBD. (A) Shematic diagram of the reaction. **(B)** 150 oligonucleotide ssDNA (40nM) was pre-incubated with hRAD51 (3uM) and hRPA (600nM), followed by addition of increasing concentrations of BRC4 DBD (500nM to 1.5uM in lanes 4 to 6). Homologous 40-mer ^{32}P-labeled dsDNA (40nM) was then added to the reaction mixtures. To evaluate the recombination mediator activity of BRC4 DBD, the samples were resolved by non-denaturing gel**. (C)** The results in **(B)** were graphed.

Figure 4. Purification of OB fold variants within the context of BRC4 and CTRB domains. (A) Chromatographic procedure designed for purification of OB fold construct variants. **(B)** Purified BRC4 OB1 CTRB, BRC4 OB12 CTRB, and BRC4 OB23 CTRB were analyzed by SDS-PAGE. BRC4 OB1 CTRB and BRC4 OB12 CTRB were co-purified with Dss1 in order to increase solubility of the BRCA2 fragments.

Figure 5. Deletion of the Tower domain attenuates dsDNA binding activity of BRC12-DBD-CTRB. (A) Purification scheme for BRC12 DBD CTRB, BRC12 DBD (delA) CTRB, and BRC12-DBD (delT) CTRB polypeptides. **(B)** Purified BRC12 DBD CTRB, BRC12 (delA) CTRB, and BRC12- BD (delT) CTRB were analyzed by SDS-PAGE. These polypeptides were co-purified with Dss1. **(C)** Purified BRC12 DBD CTRB and BRC12 DBD (delT) CTRB (150 to 900 nM in lanes 2 to 7) were incubated with 30nM ^{32}P-labeled dsDNA. The reaction mixtures were analyzed by electrophoretic mobility shift assay. **(D)** The results in **C** are graphed.

Figure 6. PALB2 purification. (A) Schematics of the known functional domains of PALB2 and the PALB2 species described in this report. **(B)** Protein purification procedures and SDS-PAGE analyses of the purified PALB2 species.

Figure 7. DNA binding activity analysis of PALB2. (A) The DNA substrates used. The prefix denotes the identity of the oligonucleotide. **(B) and (C)** Full length PALB2 (B) or the PALB2 fragment that harbors residues 1-579 (C) was incubated with mixtures of different DNA substrates (30nM each) and analyzed by non-denaturing gel. The results were quantified and graphed.

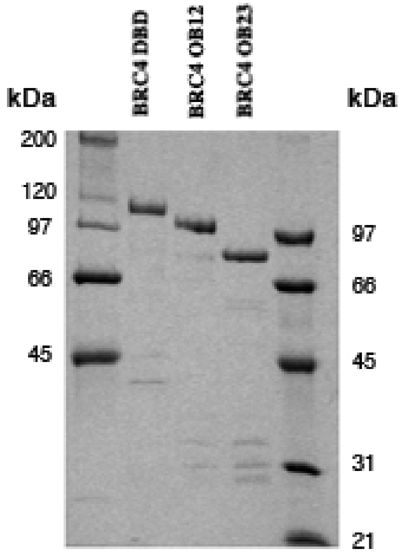
Figure 8. DNA binding activity of the N-terminal fragments of PALB2. Increasing concentrations of PALB2 fragments 1-184 (100 to 500nM in lanes 2 to 6) **(A)**, 101-184 (100 to 750 nM in lanes 2 to 8) **(B)**, and 1-43 (150nM to 1uM in lanes 2 to 9) **(C)** were incubated with the mixture of 30nM 80 nucleotide ^{32}P-labeled ssDNA and dsDNA. The results were quantified and graphed.

Figure 1

A

S100
↓
Ni
↓
GST
↓
MonoQ

B



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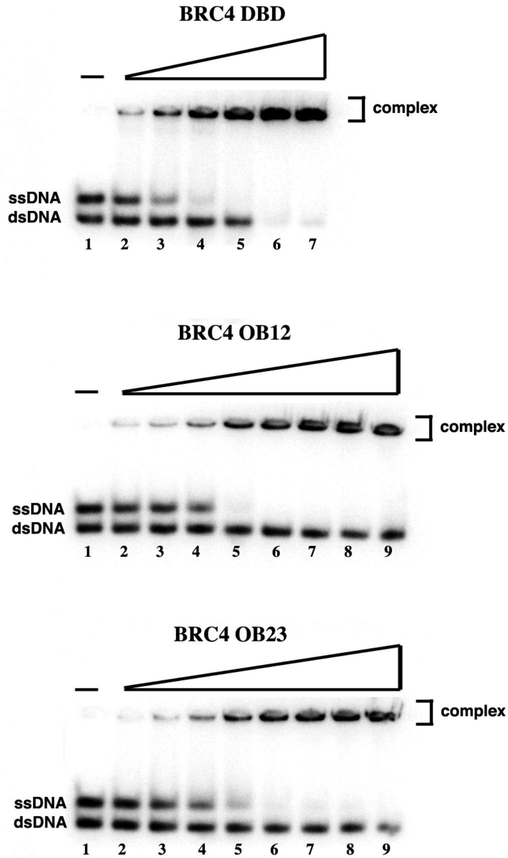
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dsDNA
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BRC4 OB12
complex
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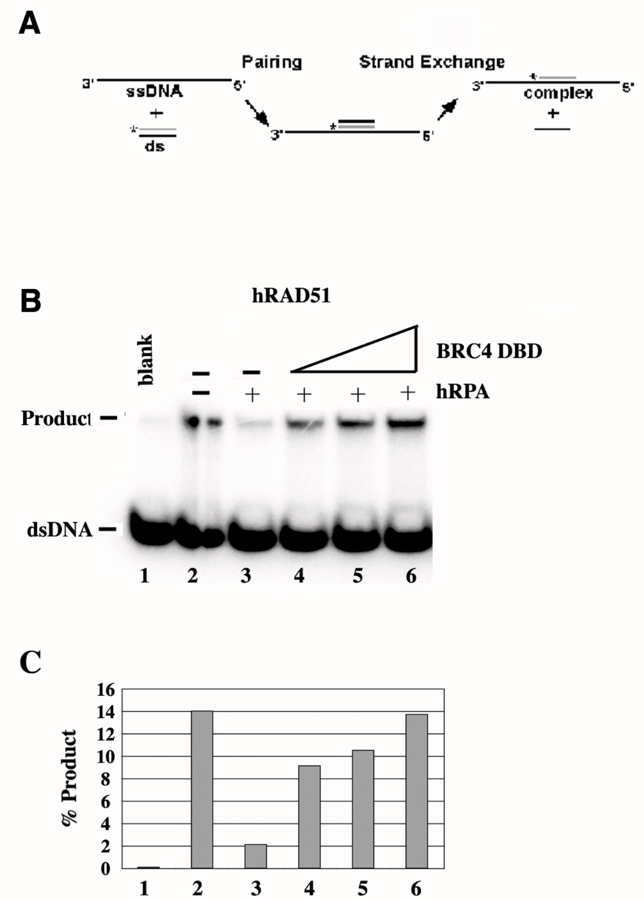
dsDNA
1 2 3 4 5 6 7 8 9
BRC4 OB23
complex
ssDNA
dsDNA
1 2 3 4 5 6 7 8 9

Figure 3



A
Pairing
Strand Exchange
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ssDNA
5'
+
ds
3'
5'
3'
complex
5'
+
B
hRAD51
BRC4 DBD
blank
hRPA
Product
dsDNA
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C
% Product
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12
10
8
6
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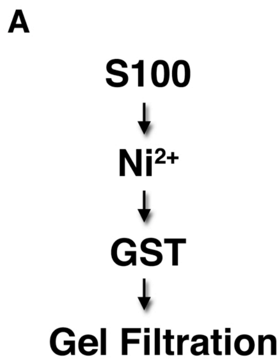
2
0
1 2 3 4 5 6

Figure 4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Ni2+
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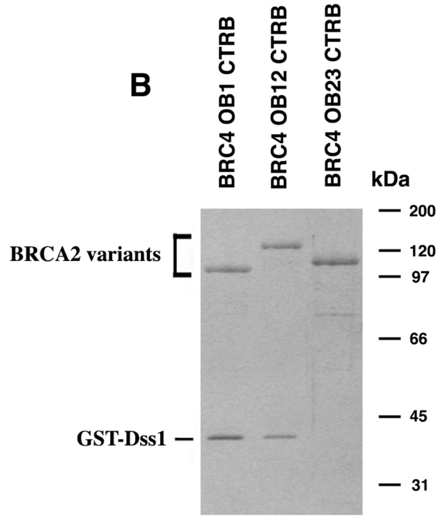
Gel Filtration


B
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BRC4 OB12 CTRB
BRC4 OB23 CTRB
kDa
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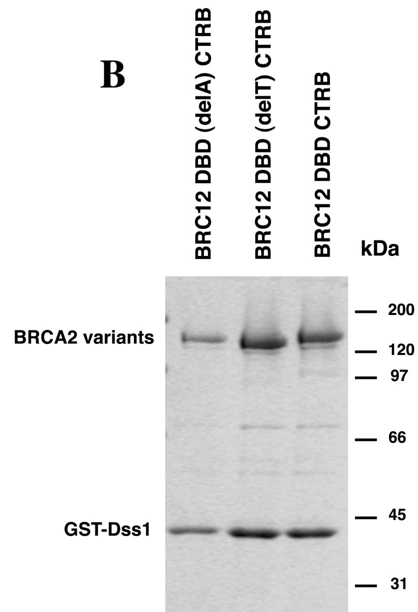
120
97
66
45
31
BRCA2 variants
GST-Dss1

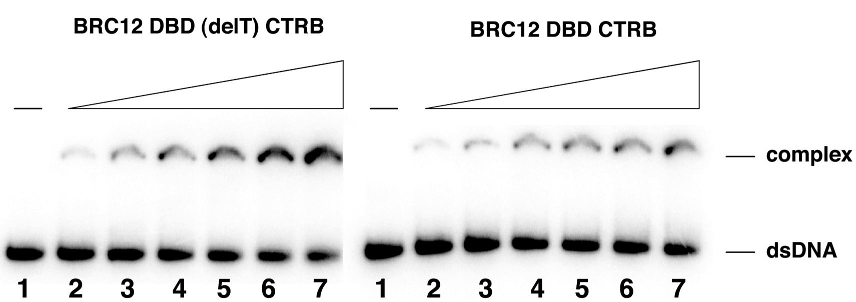
Figure 5

A

B



C



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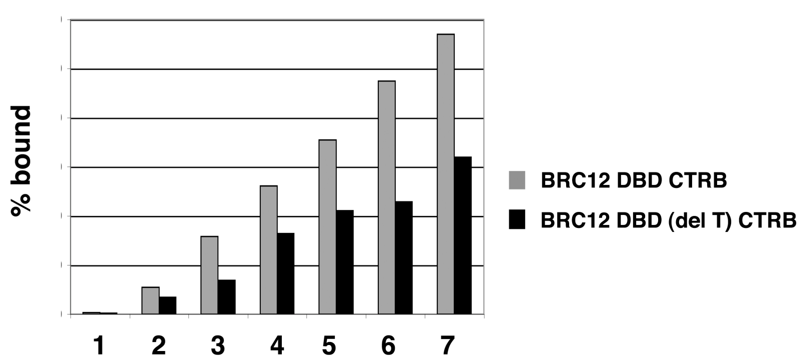

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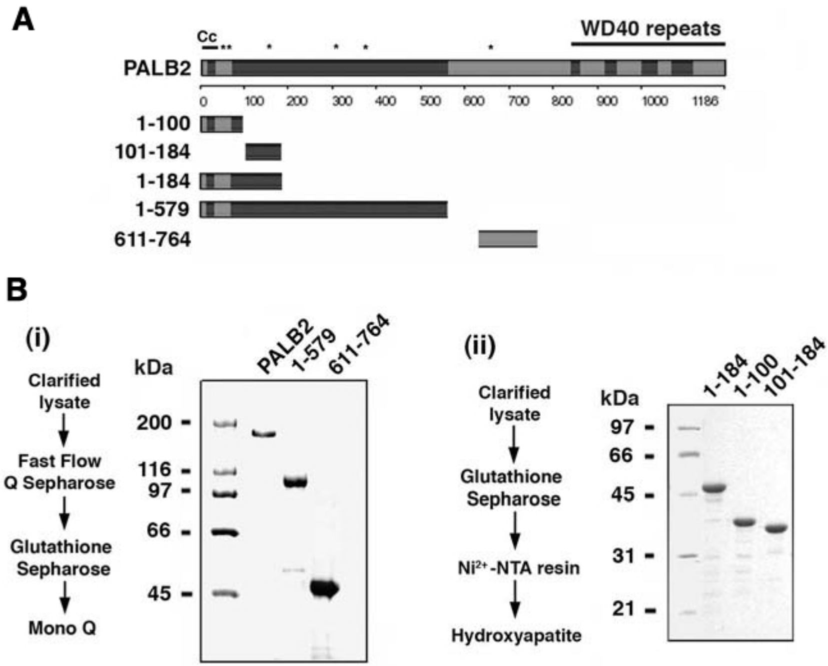
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WD40 repeats
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600
700
800
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1186
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101-184
1-184
1-579
611-764
B
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Clarified lysate
Fast Flow Q Sepharose
Glutathione Sepharose
Mono Q
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116
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45
PALB2
1-579
611-764
(ii)
Clarified lysate
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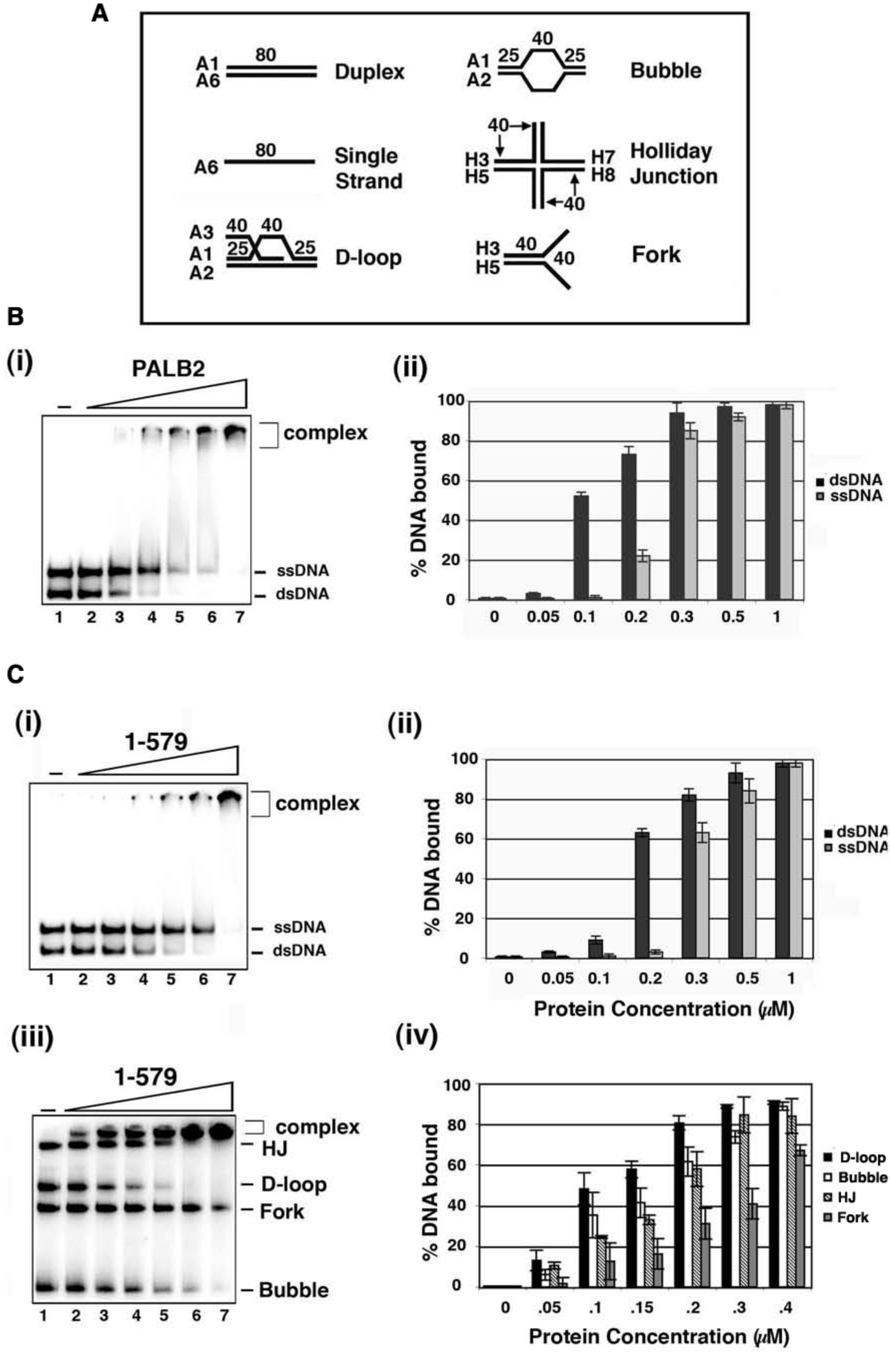
101-184

Figure 7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A2
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Bubble
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H3
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Holliday
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H5
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PALB2
complex
ssDNA
dsDNA
1 2 3 4 5 6 7
(ii)
% DNA bound
100
80
60
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20
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dsDNA
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complex
ssDNA
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% DNA bound
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Protein Concentration (μM)
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complex
HJ
D-loop
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Bubble
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(iv)
% DNA bound
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Protein Concentration (μM)
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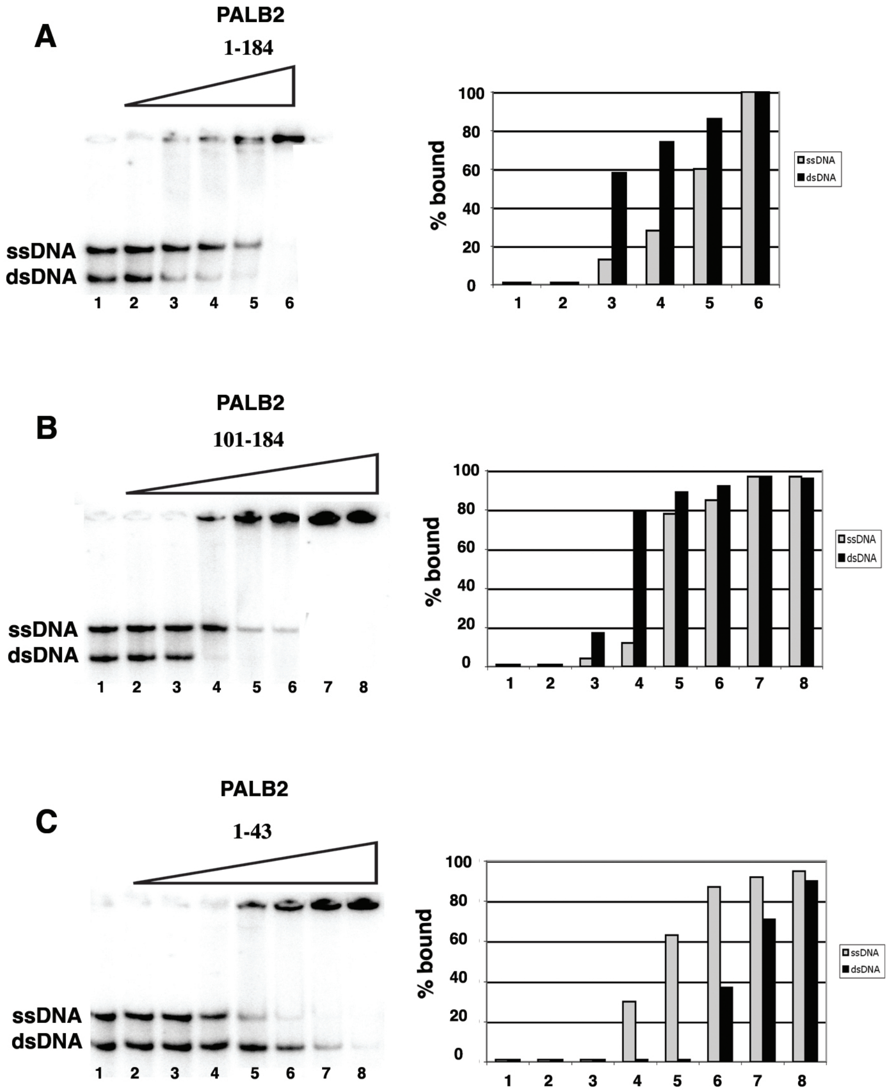
Bubble
HJ
Fork

Figure 8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